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Evaluation of Anti-Anxiety Activity of *Muntingia Calabura* Linn. Leaves Extract and Fruit Juice in Albino Mice

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***Abstract*: Anxiety disorder are the leading cause of death and disability in the world. it is an important cause of mortality around the world. Diazepam is a synthetic drug found to cause toxicity leading to severe stress in the anxiety of experimental animals. The aim of the present article is to investigate the anxiolytic effect of ethanolic extract of leaves and fresh fruit juice of *Muntingia calabura* L in mice., which may pave the way for possible therapeutic applications. Oral administration of *Muntingia calabura* L at a concentration of 200 and 400 mg/kg b.wt. and fresh fruit juice of 0.2ml showed a significant anxiolytic effect. By using various screening models, that is Open field test, Elevated plus maze test, Light and dark test and Rota rod test.**

***Keywords*: *Muntingia calabura* Linn., Ethanolic extract, Anxiolytic activity, Open field test, Elevated plus maze test, Light and dark test and Rota rod test.**

1. Introduction

Day to day stress in the humans became a major factor for many diseases [1]. Stress involves complex biochemical, neural and immunological mechanism and plays a crucial role in the progression of a variety of disease states ranging from psychiatric disorders like depression and anxiety, immune suppression, cardiovascular disease, hypertension, peptic ulcer, migraine, allergies, asthma, premature aging rheumatic disease and endocrine disorder [2]. There is a dire need from agents having neuroprotective and neuropharmacological activity enhancing learning and memory function of the brain [1].

Anxiety, a state of excessive fear, is characterized by motor tension, sympathetic hyperactivity, apprehension and vigilance syndromes. Benzodiazepines are the major class of compounds used in anxiety and they have remained the most commonly prescribed treatment for anxiety, despite the important unwanted side effects that they produce such as sedation, muscle relaxation, ataxia, amnesia, ethanol and barbiturate potentiating and tolerance [3].

Depression and anxiety are the most prevalent chronic psychiatric disorders. A number of recent studies have shown that depression predicts the onset of number of clinical conditions including hypertension, coronary heart disease, cancer, neurological disorders, hypothyroidism, as well as diabetes mellitus [4].

Herbal remedies constitute a strong component of traditional, complementary and alternative medicine. In most developing countries, herbal remedies play a critical role in the management of various diseases owing to the challenges confronting the appropriate delivery of official health care to millions of people in remote and rural communities. The World Health Organization (WHO) has advocated for the proper identification, sensible exploitation, scientific development and appropriate utilization of herbal medicines which provide safe and effective remedies in medicare [5].

There are numerous plants and polyherbal formulations claimed to have neurological activity. However, number of medicinal preparations has been used in traditional system of medicine, especially in Ayurveda for treating nerve disorders. Only a small portion of the plants as well as formulations used in traditional medicine are pharmacologically evaluated for their efficacy in nerve disorder [5]. Today there are over a number of medicinal plants that are used commercially as regulated Natural Health Products, or phytomedicines to treat mood disorders related to anxiety [6].

Medicinal plants are sources of important therapeutic aid for alleviating human ailments. Approximately 80% of the people in the developing countries depend on the traditional medicine for their primary health-care and approximately 85% of traditional medicine involves the use of plant extracts [6]. Although many drugs are available in allopathic medicine to treat neurological disorders, they produce various systemic side effects upon chronic use. Many plant products have been claimed to be free from side effects and less toxic than synthetic drugs [7].

Now, it is estimated by the World Health Organization that 80% of the world's inhabitants must rely on traditional

medicines for health care. These traditional medicines are primarily plant-based. It is also estimated that 74% of the important drugs contain active ingredients from plants used in traditional medicine. Another study of the most prescribed drugs in the USA indicated that a majority contained either a natural product or a natural product was used in the synthesis or design of the drug. All of these investigations demonstrate the importance of natural products in drug discovery [8], [9].

The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plant, through additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process. This fact has a basis in the sense that medicinal actions of plants are unique to particular plant species or groups [10].

Some plant products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters and thus have beneficial medicinal effects on human due to similarities in their potential target sites. The presence of alkaloids, tannins, cardiac glycosides, steroids, terpenoids, flavonoids, anthraquinones, phlobatannins, reducing sugars, and saponins in the plants extracts as secondary metabolites are responsible for various biological activities. The presence of flavonoids, alkaloids, terpenoids and fatty acids in the plant extract have been reported to be responsible for anxiolytic and sedative effects observed in different plant extracts [11], [12].

Therefore, several attempts have been made to treat anxiety due to stress, fear, tension, by using several antioxidant principles, antioxidant plays a crucial role in anti-anxiety ability and hence, search for crude drugs of plants origin with this has become a central focus of studies on anti-anxiety property.

In addition to this, there are reports available regarding activity of *Muntingia calabura* Linn for cytotoxic activity, antiproliferative activity, antioxidant activity and antibacterial activity.

The literature survey reveals that *Muntingia calabura* Linn. leaves and fruits have been used to treat different types of illness, the flowers and bark are used to reduce swelling in lower extremities, used to reduce gastric ulcer and flowers used to reduce stomachache, in traditional system of medicine but no scientific and methodical investigations have so far been reported in literature regarding its action on anxiety [12].

Hence the present study has been planned to evaluate the anti-anxiety activity of *Muntingia calabura* Linn. leaves extract and fresh fruit juice in albino mice.

2. Materials and Methods

A. Collection and authentication of Muntingia calabura Linn.

The *Muntingia calabura* L leaves were carefully collected from surrounding area of B G Nagara during the month of July 2018 and fresh ripen fruits were collected during the month of March 2019. The plant was identified and authenticated by renowned botanist Prof. L. B. Kulkarni, Head, Department of Botany, Sri Prabhu Arts and Science College, Surpur, Karnataka.

B. Reagents and chemicals

Diazepam (Calmpose Inj. Ranbaxy, India), Ethanol 95%. (S.D Fine Chem Ltd, Mumbai), Chloroform. (S.D Fine Chem Ltd, Mumbai), Ethyl acetate. (S.D Fine Chem Ltd, Mumbai) Dichloromethane. (S.D Fine Chem Ltd, Mumbai), Formic acid. (Thermo Fisher Scientific Pvt. Ltd, India), Toluene. (S.D Fine Chem Ltd, Mumbai), Acetone. (S.D Fine Chem Ltd, Mumbai), Acetic acid. (S.D Fine Chem Ltd, Mumbai), Methanol. (Thermo Fisher Scientific Pvt. Ltd, India), To conduct Phytochemical tests Nice Pharma chemicals was used.

C. Preparation of crude extract

The coarsely powdered leaves (500 gm) of *Muntingia Calabura* Linn were extracted with 90% ethanol by Soxhlet extraction method and the extraction temperature was maintained at 55ºC-65ºC. Extraction was carried out until disappearance of green color (approximately 48 hrs.). The extract obtained was evaporated to dryness using rotary flash evaporator and the solvent was recovered. This extract was later dried in hot air oven (55ºC ± 2ºC) and the obtained extract was stored in an air tight container and was used for anti-anxiety activities after subjecting it to preliminary qualitative phytochemical studies [13].

D. Phytochemistry

The leaves of *Muntingia Calabura* Linn are rich flavonoids, saponins, tannins, triterpenes, steroids, flavanones, flavones, flavans, squalene, triglyceride, beta-sitosterol, linoleicacid, biflavans and fruits contains many different antioxidant compounds and phenolic compounds [14]-[16].

The ethanol extract of *Muntingia Calabura* L was subjected to different preliminary chemical tests to determine the chemical constituents present in the extract.

E. Experimental animal

The animals were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 27.0±1.0° C temperature and humidity of 55±1% under 12 hours light and dark cycle. Animals were fed with standard mice feed and water ad libitum was provided. The bedding of each cages was changed thrice a week to ensure hygiene and maximum comfort for the animals. Ethical clearance for handling the animals was obtained from the Institutional Animal Ethical Committee (IAEC) prior to the beginning of the project work. All the studies conducted were approved by IAEC of SAC College of Pharmacy BG Nagara, with ethical clearance no SACCP-IAEC/27/2017 and the albino mice (Wistar) of either sex weighing 25-30 gm were selected for anti-anxiety activity. They were procured from animal house of S.A.C. college of Pharmacy, B.G. Nagara-377/PO/ReBi/S/2001/CPCSEA.

F. Dose selection

Based on literature survey we have selected leaves extract of dose 200mg/kg, p.o, 400mg/kg, p.o and 0.2ml of fresh fruit juice.

G. Experimental design

The Anti-anxiety activity of *Muntingia Calabura* L was determined in albino mice. The wistar albino mice of either sex was divided into following groups consisting of six animals each.

- Group I: Normal control mice fed with normal saline 10ml/kg (p.o).
- Group II: Mice treated with diazepam 2mg/kg (i.p).
- Group III: Mice treated with 200 mg/kg (p.o) ethanol extract of *Muntingia calabura* leaves.
- Group IV: Mice treated with 400 mg/kg (p.o) ethanol extract of *Muntingia calabura* leaves.
- Group V: Mice treated with 0.2ml (p.o) of fresh fruit juice of *Muntingia calabura.*

3. Evaluation of Anti-anxiety activity

A. Elevated plus maze test [17]

The elevated plus maze consists of two open arms and two closed arms (44 cm x 15 cm) with the open arm perpendicular to the closed one. The maze is made of wood and is located 64 cm above a black floor. Respective treatment was given to the animals and 30 minutes later, the animals was individually placed at the center of the plus maze and observed for 5 minutes.

Evaluation parameters:

1. Time spent in open arm.
2. Entries in open arm.
3. Time spent in closed arm.
4. Entries in closed arm.

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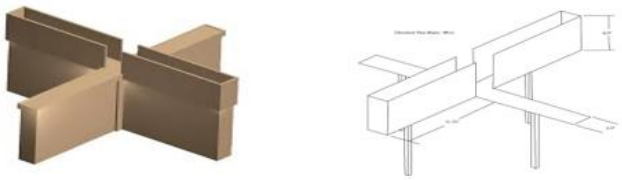

Fig. 1. Elevated Plus Maze Equipment

B. Open field test [18]

The apparatus used in this test consists of open field consists of squares (61×61cm) and was painted black except 6mm thick white lines which divide the floor into 16 squares. Open field was lighted with a 40 watts bulb focusing into the field from a height of about 100 cm. The entire room except the open field was kept dark during the experiment. Each animal was centrally placed in the test apparatus for 5 minutes.

Evaluation parameters:

1. Number of rearing.
2. Number of squares crossed.

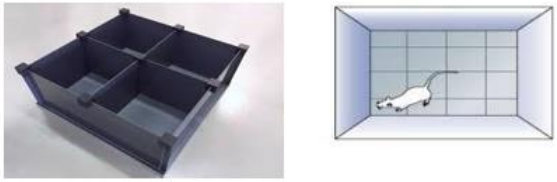

Fig. 2. Open field test equipment

C. Light and Dark Box Test [19]

The apparatus consists a rectangular box (45 X 27 X 27 cm), partitioned into two compartments connected by a 7.5 X 7.5 cm opening in the wall between compartments. Animals were placed in the center of the light compartment and observed for 5 min for the time spent in open (white/light) compartment.

Evaluation parameters:

1. Time spent (sec) in light area.
2. Time spent (sec) in Dark area.

Fig. 3. Light and dark Equipment

D. Rota Rod test [20]

The effect Muntingia calabura Linn extract of motor coordination was assessed using a Rota-rod apparatus. Rota rod apparatus consists of a base platform and an iron rod of 3 cm diameter and 30 cm length, with a non-slippery surface. This rod is divided in to four equal sections by three disks, and then enabling four mice to walk on the rod at the same time at the speed of 20 rpm observed over a period of 60 min. Intervals between the mounting of the animal on the rod and falling off of it was recorded as the performance time. There after four mice were randomly selected to determine for anti-anxiety activity. The effect on motor coordination was assessed using a Rota-rod apparatus. In brief, mice were trained to remain for 5 min on the rod rotating at speed of 20 rpm.

Evaluation parameters: Experimental mean time (5 min)

1. After 15 min of administration.
2. After 60 min of administration.

Fig. 4. Rota-Rod Equipment

4. Statistical analysis

Results of the anti-anxiety study was subjected for statistical analysis using one-way analysis of variance (ANOVA) followed by Dunnett's "t" test using GraphPad prism software. The values were expressed as mean ± SEM, n = 6, *** p < 0.01(highly significant) and *p < 0.05(significant). ns = not significant was considered as statistically significant.

5. Results

A. Preliminary phytochemical screening

The ethanol extract of *Muntingia Calabura* were subjected to different preliminary chemical tests to determine the chemical constituents present in the extract, the result has indicated that the presence of flavonoids, saponins, tannins, alkaloids, and Terpenoids compounds of which are tabulated as below (table 1)

Table 1
Result of quantitative chemical test. (+) - Present (-) – Absent.

Phytochemical Test	Leaves	Fruit
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+
Saponins	-	+
Terpenoids	-	+

1) Elevated plus maze test

The Elevated plus maze test is used to evaluate psychomotor performance and emotional aspects of rodents. Results obtained from the elevated plus maze after treatment with ethanolic extracts of *Muntingia calabura* leaves (200 and 400 mg/kg, p.o) and fresh fruit juice (0.2ml, p.o) revealed the presence of anxiolytic activity of the plant extracts. It is apparent from the table no. 2 that *Muntingia calabura* leaves extract shown dose dependent anxiolytic activity. Results of the present study indicates that animals treated with leaves extract, fruit juice and diazepam spent more time in open arm rather than closed arm in which animals administered with saline spent more time. Similar was the case with regard to the time spent in open arm and closed arm (table 2). Among the samples studied, *Muntingia calabura* leaves extract at the dose of 400 mg/kg shown significant anxiolytic activity in terms of number of entries in comparison with the normal animals and in addition its activity was comparable with that of the reference standard diazepam. Leaves extract with the dose of 200 mg/kg was shown least anxiolytic effect among all the tested samples in terms of entry and time spent in two different arms. In the present study, increase in the entry of animals as well time spent in open arm are the most representative indices of anxiolytic activity. In accordance with the present results, *Coriandrum sativum* fruit extracts also shown anxiolytic activity by frequently entering and staying the open arm for long time [21].

2) Open field test

Generally, the open field test is used to evaluate the animal emotional state. The open field model examines anxiety related behavior characterized by the normal aversion of the animal to an open and brightly lighted area. Thus, animals removed from their acclimatized cage and placed in environment express anxiety and fear by showing alteration in some parameters like square crossing and rearings. Anxiolytic drugs will help in reducing such fearful behavior of animals in open field. The results obtained from the open field study is presented in the table no. 3. It is evident from the table no. 3 that animals treated with *Muntingia calabura* leaves extract at a higher dose of 400 mg/kg and fresh fruit juice (0.2ml) showed significant increase in the number of rearings and number of squares crossing in comparison with the vehicle treated group, which clearly indicates the anxiolytic-like effect of the plant extract. The anxiolytic effect exhibited by the higher dose of *Muntingia calabura* leaves extract was significantly higher than the effect shown by the reference standard drug diazepam (table no.3) The anxiolytic activity of the tested sample was in the order of *Muntingia calabura* leaves extract 400 mg > diazepam 2 mg > *Muntingia calabura* leaves extract 200 mg > fresh juice.

3) Light and Dark Box Test

In the light and dark model, anxiety is generated by the

Table 2
Effect of ethanol extracts of *Muntingia calabura* Linn leaves and fresh fruit juice on elevated plus maze test in mice

Group	Treatment	Dose	Number of entries (mean± SEM)		Time spent in sec (mean± SEM)	
			Open arm	Closed arm	Open arm	Closed arm
1	Saline	10 ml/kg	7.45±1.36	15.83±2.56	123.16±7.02	177.67±7.76
2	Diazepam	2 mg/kg	19.16±1.1***	2.17±1.17***	226.50±9.20***	73.17±6.53***
3	Leaves extract	200 mg/kg	9.33±1.13*	7.50±1.38*	173.67±4.54*	126.84±7.60*
4	Leaves extract	400 mg/kg	14.67±1.3***	4.67±1.36***	210.33±8.47***	90.83±4.11***
5	Fruit juice	0.2 ml	11.16±1.4***	5.71±1.37***	182.83±5.45***	118.17±4.4***

Value are expressed as mean ± SEM (n = 6). *** P < 0.01 and * P < 0.05. ns = not significant

Table 3
Effect of ethanol extract of *Muntingia calabura* Linn leaves and fresh fruit juice on open field test in mice

Group	Treatment	Dose	Number of squares crossed	Number of rearing
1	Saline	10ml/Kg	7.2±0.62	6.67±0.35
2	Diazepam	2mg/kg	9.3±0.95***	7.46±0.41
3	Leaves extract	200mg/kg	7.3±0.68	7.08±0.38
4	Leaves extract	400mg/kg	9.9±0.73***	10.32±0.53***
5	Fresh fruit juice	0.2ml	8.2±0.78*	8.92±0.44*

Value are expressed as mean ± SEM (n = 6). *** P < 0.01 and * P < 0.05. ns = not significant

Table 4
Effect of ethanol extract of *Muntingia calabura* Linn leaves and fresh fruit juice on light and dark box test in mice

Group	Treatment	Dose	Time spent in light area (sec)	Time spent in dark area (sec)
1	Saline	10ml/Kg	121.83±4.70	196.50±7.96
2	Diazepam	2mg/kg	192.03±7.16***	117.93±4.83***
3	Leaves extract	200mg/kg	138.50±5.61*	177.16±5.03*
4	Leaves extract	400mg/kg	212.83±8.89***	94.50±4.08***
5	Fresh fruit juice	0.2ml	155.16±4.99*	148.33±7.25*

Value are expressed as mean ± SEM (n = 6). *** P < 0.01 and * P < 0.05. ns = not significant

Table 5
Effect of ethanol extract of *Muntingia calabura* Linn leaves and fresh fruit juice on rota rod test in mice

Group	Treatment	Dose	Experimental mean time (5min) (s)	
			After 15 min Administration	After 60 min of Administration
1	Saline	10ml/Kg	229.88±8.90	138.50±7.81
2	Diazepam	2mg/kg	185.50±7.76*	87.16±3.60***
3	Leaves extract	200mg/kg	223.66±3.14*	116.33±5.57*
4	Leaves extract	400mg/kg	159.52±6.02***	64.83±4.45***
5	Fresh fruit juice	0.2ml	211.51±4.46*	104.17±7.13*

Value are expressed as mean ± SEM (n = 6). *** P < 0.01 and * P < 0.05. ns = not significant

conflict between the tendency to explore and the initial tendency to avoid the unfamiliar and can be evaluated according to the number of transitions in to and the time spent in the light chamber, where in increase in these parameters is considered to reflect anxiolytic-like properties. Results of light and dark model is represented in table 4. It evident from the table no. 4 that animals treated with samples spent more time (seconds) in light area in comparison with the saline where in animals spent more time in dark area. This clearly indicates the anxiolytic effect of the treated samples in comparison with control sample. The order of time spent in light area was *Muntingia calabura* leaves extract 400 mg/kg > diazepam > fresh juice > *Muntingia calabura* leaves extract 200 mg/kg. In accordance with the present results, *Urtica urens* extracts also shown anxiolytic activity by Spending more time in light area rather than dark area [22].

4) Rota rod test

Rota rod test was first introduced to screen the assay of neurotoxicity of anticonvulsants and later was reported to predict motor dysfunction produced by centrally acting drugs to determine possible alterations in the motor coordination ability of the animal, often caused by the use of sedative and antipsychotic drugs. In this model, the difference in the falling time from the rotating rod between the vehicle and sample treated groups is taken as an index of muscle relaxation. The skeletal muscle relaxation together with taming or calming effect, also reduces anxiety and tension. It is evident from the table no. 05 that after 15 min, animals administered with saline fall off from the rota rod after a long duration of 229.88±8.90 sec followed by *Muntingia calabura* leaves extract 200 mg/kg body weight (223.66±3.14), fruit fresh juice (211.51±4.46), diazepam (185.50±7.76) and *Muntingia calabura* leaves extract 400 mg/kg (159.52±6.02). After 60 min, the trend of 15 min drug administration was continued but the time taken for falling off from the rota rod was reduced. Similar type of results was also reported for *Erythrina mysorensis* extracts in which the extract shown anxiolytic activity with the reduction in the fall off time in mice.[23] The present result was also in contrary to the findings of *Urtica urens* in which the falling off time was increased with the increase in the duration of drug administration [22].

6. Discussion

Earlier reports on the chemical constituents of plants and their pharmacological activity suggest that plants containing flavonoids, alkaloids, phenolic acids, essential oil, saponins and tannins possess anxiolytic activity.[12] Even in the present study, preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins and terpenoids (table no. 01). The found anxiolytic and muscle relaxation potency of the *Muntingia calabura* leaves extract and juice extract might be due to the presence of these phytochemicals. In addition, the significant activity might be due to the possible synergistic action of these phytochemicals present in the leaves extract and juice.

7. Conclusion

Muntingia calabura L leaves has been traditionally used to treat various disorders however no scientific studies are available regarding its anxiolytic activity. The present study showed that *Muntingia calabura* L. extract possess significant anxiolytic effect in terms of modifying various anxiety-related behavioral parameters which are evaluated through various animal models like light and dark test, rota rod test, elevated plus maze test and the open field test in mice. The found anxiolytic effect of *Muntingia calabura* L. leaves extract and fresh fruit juice was comparable with the effects of standard diazepam. The outcome of the present study provides the scientific evidence for the traditional use of *Muntingia calabura* L. by Indian people to treat anxiety. Results of the present study also suggests the potential clinical application and commercial utilization of *Muntingia calabura* L. in the management of anxiety disorders.

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